Global Journal of Fertility and Research

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Dates: **Received:** 06 May, 2016; **Accepted:** 25 May, 2016; **Published:** 26 May, 2016

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Keywords: Embryo morphokinetics; Aneuploidy; Embryo genotyping; Sequencing

Letter to Editor

Novel Scientific Methods and Technology in the Reproductive Medicine

Abstract

Objectives: The objective of this review is to provide synopsis on the latest technology and scientific achievements that have found application in the reproductive medicine.

Results: The application of a novel time-lapse technology into reproductive medicine promotes; 1) the non-invasive observation of early embryogenesis *in vitro* and provides an opportunity for a more accurate understanding of developmental dynamics of the early embryo and it's consequence for the further successful development, 2) the establishment of promising morphokinetic parameters of the developing embryo *in vitro* and identification of the embryos with a high implantation potential. Determination of the chromosomal abnormalities (aneuploidy) in the pre-implanting embryo using preimplantation genetic testing technology (PGD) increases the chances of selecting a genetically normal and therefore implantable embryo for the transfer, since the embryonic mortality is strongly affected by the genetic status of the embryo.

Conclusion: Merging the state-of-the-art methodologies and innovations in reproductive science boosts the efficiency of the infertility treatment and advancing animal's reproductive performance.

Abbreviations

IVF: *In vitro* Fertilization; PGD: Preimplantation Genetic Testing; aCGH: Array Comparative Genomic Hybridization; SNP: Single Nucleotide Polymorphism; PCR: Polymerase Chain Reaction; NGS: The Next Generation of Sequencing; ART: Assisted Reproductive Technologies; PCOS: Polycystic Ovary Syndrome; eSET: Single Embryo Transfer;

Introduction

By combining novel scientific and technology accomplishments it is possible to create the-state-of-the-art options for the infertility treatments in humans, and to deliver advancement in animal breeding activities. Embryo creation *in vitro* with embryo transfer allows to overcome many aspects of human infertility. The most popular criteria to assess the quality of embryo in the clinical and veterinary practice are based on the evaluation of embryo's morphological quality at the time of transfer [1,2]. This approach is extremely subjective and inadequate, because a snapshot morphological assessment of the embryo has a limited success compared to the evaluation of the kinetic changes and embryo morphology over the time of its development [3]. The study of Wong et al. [4], demonstrated that two morphologically similar embryos, being at the same developmental stage at the time of point assessment undergone a completely different developmental process when the kinetics of the embryo was taken into consideration. A recent study of Walls et al. [5], involving the use of time-lapse technology, showed that embryos from hyperandrogenic PCOS women were significantly delayed at early stages of the development, when compared to embryos of non-PCOS regularly cycling women. Another paper [6], documented that embryos derived from a woman undergoing ovarian stimulation with the flexible GnRH antagonist protocol underwent faster with the earliest cleavage, when compared to embryos derived from a woman undergoing the long GnRH agonist protocol. Hence, it is evident that the regiment used for the ovarian stimulation was associated with the embryo cleavage anomaly.

Previously, a clear relationship between the time of first cleavage and embryo developmental competence has been demonstrated. The zygotes cleaving earlier after insemination are more likely to reach the blastocyst stage than their later cleaving counterparts [7,8]. This phenomenon is common in many species, e.g. rhesus monkey [9], human [10,11] and buffalo [12]. The factors that control the time of the first cleavage are unclear. However, the gene controlling the rate of preimplantation cleavage divisions and subsequent embryo survival has been identified in mice [13].

Sugimura et al. [14], demonstrated that using multiple predictors such as; timing of the first cleavage, the number of blastomeres at the end of the first cleavage, presence or absence of multiple fragments at the end of the first cleavage, the number of blastomeres at the temporary developmental arrest (lag-phase) during the fourth or fifth cell cycle and oxygen consumption at the blastocyst stage, allows to objectively and reliably select healthy IVF embryos that resulted in a successful pregnancy. It is important for the clinical practice to determine the embryo with the highest potential for implantation and development, as the selective single embryo transfer (eSET) is becoming increasingly popular with a view to reduce the risk for multiple pregnancies effectively. Thus, selecting an optimal embryo for transfer into uterus is a major challenge in assisted reproductive technology. Therefore, novel criteria that will allow evaluate the embryos objectively and reliably are needed to advance the IVF

technology. Such an opportunity is provided by a newly emerging non-invasive Time-Lapse Microscopy technology.

Morphokinetics of the embryo

The time-lapse technology is a tool for a non-invasive continuous monitoring of the development of an individual embryo *in vitro*, embryo developmental kinetics, the number of blastomeres, symmetry of cell division, and the degree of cytoplasmic fragmentation. The principal objective of the method is to establish non-invasive criteria that could predict not only blastocyst qualities but also their potential for implantation into uterus after transfer. The additional benefit of the application of the time-lapse system entails avoiding removal of the embryos from optimal culture conditions, thus decreasing the risk of deleterious effects during their transfer between the incubator and the microscope throughout daily observations. The time- lapse system is becoming increasingly popular in human IVF as a number of embryo selection methods based on time-lapse imaging have been recently reported for humans.

Meseguer et al. [15], developed a hierarchical multivariable embryo selection method that categorizes embryos into 10 grades using morphology and timing parameters (time to 5 cells, synchrony in divisions from two-cell to four-cell, and duration of the second cell cycle), and based on the already known implantation data from day 3 embryo transfers. Later, this model was tested retrospectively and it was found that the classification results correlated with the pregnancy rates [16].

Conaghan et al. [17], developed the embryo classification model which, when used in combination with traditional embryo morphology assessment allowed to predict embryo developmental potential at the cleavage stage. This classification model was based on a two-cell division timing parameters, specifically the time between the first and second mitosis, or duration of the two-cell stage and the time between the second and third mitosis, or duration of the three-cell stage. Each of the parameters correlated with embryo development, implantation potential, or both [15,18-22].

Recently, Milewski et al. [23], created a multivariate predictive model for embryo development into a blastocyst. He designed univariate and multivariate logistic regression analyses on the basis of the results obtained from culturing the embryo in the EmbryoScope®. This multivariate predictive model is based on the time of divisions from two to five blastomeres and intervals between the second and third division.

Dal Canto et al. [24], investigated the morphokinetics of embryos that develop to blastocysts and their ability to implant. The study demonstrated, that cleavage time to the 7- and 8 cell stages and relative intervals from the 4- to 8- cells stage, and also from the 5- to 8- cells stage were statistically different from embryos arresting after the 8-cell stage. The expansion of blastocysts correlated with all cleavage times from the 3-cell stage onwards. Moreover, implanted embryos usually achieved the 8-cell stage earlier than those that did not implant.

Basile et al. [25], reported the embryo selection method based on embryo developmental kinetics, ranking the probability of day 3 embryos being chromosomally normal. However, this method has not been validated yet using prospective independent clinical data.

On the other hand, the time lapse –microscopy method is rarely used in the selection of embryos for transfer in the farm animals [26]. This might be due to the lack of established and reliable morphokinetic selection criteria for embryos of different animals.

Genetic assessment of the embryo

It is well known that fertility in mammals is strongly affected by chromosomal abnormality, which represents one of the major contributors to the developmental failure associated with a reduced embryo developmental potential [27], impaired embryo viability [28], as well as embryonic death and abortion [27]. A chromosomally abnormal embryo or fetus will never result in a normal healthy pregnancy or a baby. Most of the reports about chromosome abnormalities in human embryos and domestic animals describe numerical aberrations (aneuploidy – defined as the gain or loss of the entire chromosome) comprising haploidy, polyploidy triploidy and tetraploidy and mixoploidy, with a frequency ranging from 5 % to 39 % depending the species [27,29] and as high as 60% [30], contributing to the vast majority of pregnancy losses in both natural and assisted reproduction technology (ART) conceptions.

It has also been demonstrated that the percentage of chromosomal abnormality is considerably influenced by the maturation environment of the oocytes [31], as well as post-fertilization culture condition for *in vitro* produced embryos [32]. Therefore, *in vitro*-derived embryos have a higher rate of chromosomal abnormalities than *in vivo* counterparts [33]. They are marked by a lower quality and viability to term than those produced by the *in vivo* method, even when the same donor was used [34]. King [27] suggested that approximately one quarter of the abnormalities (haploidy, polyploidy, mixoploidy) may be attributed to errors in meiosis with the remaining three quarters occurring around the time of fertilization and early embryonic development. Similar results have been reported for pig [35], horse [36], goat [37], sheep [38] and humans [39].

Embryos produced after *in vitro* fertilization (IVF) can be tested for the correct number of chromosomes using the preimplantation genetic diagnosis (PGD). During this process, a biopsy is performed usually on the embryos on the 5 or 6 day of development (blastocyst stage). Previously reported aneuploidy rate in humans and animals was assessed primarily by the fluorescent in situ hybridization (FISH) method. However, the FISH method is marked by a number of significant limitations (quality of chromosomes spreading, high possibility of losing some chromosomes during fixation, overlapping chromosomes) and most importantly, by the inability of examining the whole set of chromosomes in the same sample.

Currently a range of molecular genetic technologies based on the use of microarrays (e.g. comparative genomic hybridization (aCGH) or analyses of single nucleotide polymorphism [40], or quantitative PCR [41], or next generation of sequencing (NGS) [39], may be applied to screening the embryos for multiple markers of different diseases or mutations or the copy number of all pairs of chromosomes from biopsies of the pre-implanting embryos. The main obstacle in testing the preimplantation embryo constitutes an extremely limited

amount of tissue available for the analysis. Therefore, there is a trade-off between the successful testing and the survival of the embryo. Generally, the lower number of cells or starting DNA quantity in the biopsy sample, the lower success of the whole genome amplification (WGA), yet a higher survival rate of the embryos and vice versa. Thanks to a recent significant progress, the WGA technology may currently be applied successfully to limited genomic DNA quantities such as trophectoderm biopsy samples [42]. Campbell et al. [43], established the aneuploidy risk classification model for blastocyst stage embryos. For this purpose, she used data from genetic testing of the blastomere or trophectoderm biopsy and morphokinetic parameters of a developing human embryo. This model is based on the starting time of blastulation and the time needed to acquire a fully expanded blastocyst stage. The model was tested in a retrospective study and revealed that classification results corresponded to the probability of implantation [44].

Accurate determination of the aneuploidy in the pre-implanting embryo will promote the selection of embryos with a high implantation potential for the embryo transfer. The biological significance of eliminating the chromosomally abnormal embryos has been clearly illustrated in humans. Transferring a single blastocyst with the correct number of chromosomes contributed to a considerable increase in the probability of achieving pregnancy and reducing miscarriages [45,46]. Therefore, screening the embryo for chromosomal aneuploidy in cases of advanced maternal age or known parental translocation in humans is becoming a quite common procedure in several countries over the world to reduce miscarriage and increase live birth rate.

An accurate assessment of the frequency of chromosomal anomaly in embryos of farm animals is much more limited compared to humans, due to the lack of a rigorous system for monitoring embryo/fetus development during the prenatal period. In addition, the samples of miscarriages or abnormally born animals are rarely sent to cytogenetic laboratories for examination. As a result, the precise frequency of chromosomal anomaly in developing animal embryos still remains uncertain. Therefore, the verification of the number of chromosomally abnormal animal embryos is needed.

The innovations in molecular genetics and molecular biology techniques have also provided benefits in the sector of animal breeding. Those technologies have already been applied for single qualitative gene tests, such as embryo sex and several lethal genes (BLAD, CVM). The value of molecular information in making decisions of assisted breeding has already been demonstrated, particularly through the use of the marker-assisted selection as well as in case of monitoring population structure and obtaining information on the history and development of populations. However, embryo genotyping is another tool that may facilitate the assessment of genetic breeding values of the embryo even before implantation. Therefore, marker-assisted embryo selection may be applied at the stage of the pre-implanting embryo from the blastocyst trophectoderm biopsies. The biopsies from the pre-implanting embryos may be screened for multiple markers of breeding and/or economic importance. Genotyping has now become an available tool for the most livestock species and is used routinely in marker-assisted breeding selection programs. Applying this technology, breeders may produce animals of a high genetic merit in a much shorter time in comparison to a traditional selection method that based upon progeny testing, where the genetic merit of candidate bulls is judged either by a large number of daughters being milked on the farms or by the quantity of beef obtained from slaughtered cattle. The potential benefits of embryo-based genomic selection include 1) a direct link between the genetic evaluation and the genome, 2) an increased accuracy with a minimal rate of inbreeding, 3) an increase in the rate of genetic progress through accelerated intensity of selection of males from the best females, 4) identification of desirable DNA fragments in commercial populations that may be selected for special interest, 5) the opportunity to overcome or reduce sex and age limitations for traits that can only be measured late in life, and 6) the possibility to establish a bank of significant value embryos.

Sexing embryo

Recent advancement in the field of genetics, genetic diagnosis, embryo biopsy and pre-implantation genetic diagnosis (PGD) has opened up a new world for sex selection in the embryo prior to transfer. Nearly 95% embryos may be sexed by Y-specific chromosome probe using the polymerase chain reaction (PCR) [39], or the fluorescent in situ hybridization (FISH) [47]. A considerable improvement in the PCR technique such as multiplex PCR [48] or in the loop-mediated isothermal amplification method, constituting a new generation of innovative gene amplification techniques, allows for a rapid sex determination in the embryo [49], with 100 % reported accuracy of sex prediction [50]. Therefore, the PGD constitutes a procedure with the success rates of sex predetermination in embryo being as high as 99.9%. Knowledge of the gender of the animal embryo before placing it into recipient's uterus will provide opportunities for customizing animals for different markets (cows or bulls farms, milk or meat farm) and advancing farm management. Although, sex determination in human embryos remains a controversial topic, it may be advantageous in cases of diseases linked to sex chromosomes. By selecting only unaffected embryos for the transfer it eludes the birth of unhealthy baby.

Cryopreservation

Cryopreservation of embryos has always played an important role in the assisted reproductive technologies. It allows to increase effectiveness of the IVF cycle and to decrease the number of fresh embryos to transfer. Recent significant advancement in the vitrification process produced a highly effective commercial freezing method for the oocytes and blastocysts, including blastocysts subjected to the biopsy procedure [46]. Currently, this method is widely used in human IVF and has resulted in the birth of more healthy babies after the transfer of vitrified blastocysts [51]. Additionally, the successful oocyte vitrification resulted in considerable increase in the number of normal births when compared to any other cryopreservation methods [52].

To improve the survival of the embryo it was also suggested that an increased volume of blastocoelic fluid in the expanding blastocysts may be associated with a poor survival after cryopreservation due to potential ice crystal formation. The study of Desai et al. [53], revealed that reduction of the fluid volume using either mechanical or laser technique may reduce DNA damage and enhance post-warming re-

expansion and cell proliferation in expanding blastocysts. Frozen embryos may be stored in liquid nitrogen for a long time without affecting their viability and causing a genetic change [54], and may be used in the future attempts of pregnancy in case of both humans and animals. Therefore, the application of this technology entails a more economical use of embryos and a more efficient management of patients IVF cycles. Furthermore, the breeders may more successfully disseminate the smart gene or desirable genetics around the world, what makes embryos freezing a vital tool for the international trade subjects. The use of frozen animal embryos allows for: 1) efficient use of donors and recipient, 2) achieving the genetic progress at low cost, 3) comparison of the values of the embryo and its transport towards animals standing, 4) transferring some embryos and keeping the rest until record of analyses of produced offspring will be available, 5) improvement in disease control, 6) creating valuable embryo banks of livestock, 7) customizing animals to different markets (females & bulls farms).

Conclusion

Wide application of the innovative and modern technologies into reproductive medicine, constitute a tool for selecting the most developmentally competent embryos for transfer and subsequent delivery of healthy babies or superior genotypes in the population of livestock. The efficient vitrification of the excess embryos may lead to substantial savings for the couples struggling with infertility and/or allow breeders to more efficiently disseminate the smart genes or desirable genetics worldwide.

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Citation: *Wiesak T, Goryszewska E (2016) Novel Scientific Methods and Technology in the Reproductive Medicine. Glob J Fertil Res 1(1): 001-005.*